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Characterization of the glutamate dehydrogenase isoenzyme system in germinating soybean

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Abstract

Protein extracts from different soybean (*Glycine max* L. Merr. cv. Williams) organs contained four glutamate dehydrogenase (GDH) isoenzymes (EC 1.4.1.2–1.4.1.4) when native polyacrylamide gels were stained for NAD-dependent activity. The four isoenzymes were designated GDH0, GDH1, GDH2 and GDH3. A combination of NAD- or NADP-specific GDH stains and immunoblot analysis of native polyacrylamide gels was used to characterize the four GDH isoenzymes. Isoenzymes GDH0, GDH1 and GDH2 demonstrated both NAD- and NADP-dependent activity, while GDH3 only had NAD-dependent activity. Based on the intensity of the different stains, GDH1 and GDH2 had greater activity with NAD than with NADP, while GDH0 had less activity with NAD than with NADP. Both GDH2 and GDH3 cross-reacted with rabbit serum raised against grape leaf NAD(H)-GDH, but the reaction was more intense with GDH3 than GDH2. Tissue print analyses were used to demonstrate the tissue-specific accumulation of GDH3 activity throughout the axis during germination in the dark. Negative controls (GDH stain solution lacking glutamate) and immunodetection with antiserum to grape NAD(H)-GDH validated the specificity of the NAD-GDH tissue print analyses. NAD-GDH activity was most abundant in the phloem of the hypocotyl hook

Abbreviations: β-ME, β-mercaptoethanol; GDH, glutamate dehydrogenase; NAD(H)-GDH, NAD(H)-dependent GDH; NADP(H)-GDH, NADP(H)-dependent GDH; PMSF, phenylmethylsulfonyl fluoride; PPFD, photosynthetic photon flux density; PVP, polyvinylpyrrolidone.

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with diffuse activity throughout the cortex. In addition, NAD-GDH activity was apparent in the epidermis of the hypocotyl hook. In the region of the hypocotyl below the hook and above the root hairs, enzyme activity was evident throughout the cortex and epidermis and absent in the vascular tissues. In the root, NAD-GDH activity was localized exclusively in the epidermal layer, particularly in the region of the functional root hairs. All other tissue types in this region of the root, including the xylem, phloem and cortex, were devoid of GDH activity. In emerging lateral roots, NAD-GDH activity was localized in the root tip and epidermis. Since only GDH3 was detected in these tissues, the data suggest that GDH3 may play a unique role in the mobilization of carbon or nitrogen in germinating seedlings.
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1. Introduction

Glutamate dehydrogenase (GDH; EC 1.4.1.2–1.4.1.4) catalyzes the pyridine nucleotide cofactor-dependent reversible reductive amination of α-ketoglutarate to form glutamate. In most plants, there are distinct GDH isoenzymes that preferentially utilize either NAD(H) or NADP(H), but some GDH isoenzymes have been shown to use either cofactor (for review see [1,2]). In general, NAD(H)-GDH isoenzymes have been identified throughout the plant, while NADP(H)-GDH isoenzymes are usually associated with green tissues. GDH isoenzymes have been well characterized in several plant species. The most extensively characterized NAD(H) isoenzyme systems are in grape [3–7] and *Arabidopsis* [8–11]. In both species, there are seven NAD(H)-GDH isoenzymes that can be readily resolved by native polyacrylamide gel electrophoresis (PAGE).

The isoenzymes are composed of two subunits, designated α and β, of different molecular size (between approximately 42 and 43 kDa), that can be resolved by sodium dodecyl sulfate (SDS)-PAGE [5,11]. The two subunits combine in different ratios to form seven enzymatically active hexameric complexes. This architecture appears to be well conserved in the plant kingdom, since similar results have been demonstrated in avocado [12]. However, some plants, such as soybean, do not appear to follow this pattern. Soybean NAD(H)-GDH isoenzymes do not resolve into seven distinct bands [13,14]. Based on their electrophoretic mobilities, three NAD(H)-GDH isoenzymes (GDH1, GDH2 and GDH3) have been identified in protein extracts of 5-day-old soybean seedlings germinated in the dark [14]. Likewise, three NAD(H)-GDH isoenzymes were identified in developing soybean seeds obtained 35 days after flowering [13,15]. A summary of the results from the combined studies suggest: (1) GDH1, the fastest migrating isoenzyme, is specific to the cotyledonary tissue in both developing seeds and germinating seedlings; (2) GDH2 is associated with both cotyledons and leaves; (3) the slowest migrating isoenzyme, GDH3, is in roots and hypocotyls.

Recently, one of the soybean isoenzymes, GDH3, was purified to homogeneity from hypocotyls and roots of 5-day-old soybean seedlings germinated in the dark and was physically and biochemically characterized [14]. GDH3 was associated with intact mitochondria. The isoenzyme had two pH optima: one for reductive amination at pH 8.0 and a second for oxidative deamination at 9.3. Estimates of GDH activity were 12–50-fold higher in the direction of reductive amination when compared to estimates of enzyme activity in the direction of the oxidative deamination reaction conducted at the same pH. Interestingly, the amination reaction was stimulated 2–17-fold by divalent cations, and the reaction was most responsive to changes in Ca^{2+}. Conversely, the deamination reaction was not affected by changes in cation concentrations (F.J. Turano, unpublished results). GDH3 had a cofactor preference for NAD(H) over NADP(H). The apparent K_m values for glutamate, α-ketoglutarate, ammonium and NAD(H) were determined at different pH values. The results from those investigations, in conjunction with other studies [13,15], provide detailed information on the physical and biochemical properties of GDH3. The apparent K_m values for the substrates and cofactors [15] and general information on the physical characteristics of GDH1 and GDH2 [13] have also been reported.

Compared to the GDH isoenzyme systems in grape and *Arabidopsis*, the soybean NAD(H)/NADP(H)-GDH isoenzyme system has not been studied in detail. As part of a continuing effort in the laboratory to understand the physiological role of NAD(H)/NADP(H)-GDH isoenzymes in plants, we are focusing our efforts on two different systems, soybean [14] and *Arabidopsis* [11]. In this study, we utilized enzyme-specific stains with different cofactors and immunoblot analysis of native gels to characterize the different soybean GDH isoenzymes. In addition, we used similar techniques to localize NAD-GDH activity in the axis of soybean seedlings germinated in the dark.

2. Materials and methods

2.1. Plant material, crude and purified protein extractions

Soybean (*Glycine max* L. Merr. cv. Williams) seeds were germinated on paper towels moistened with deionized water in the dark for 5 days at 25°C under aseptic conditions as described by Turano et al. [14]. After 5 days, cotyledons, hypocotyls and roots were separated, and protein samples were extracted from each organ. For the light-grown samples, five seeds were germinated in plastic covered pots (20 × 10 × 6 cm, length × width × depth) filled with vermiculite moisten with sterile deionized water. After 5 days the plastic covering was removed. Plants were maintained at 25°C, 60–70% relative humidity under cool white lights (500 mmol PPFD $m^{-2} s^{-1}$) in a 16-h light/8-h dark cycle. The plants were irrigated with deionized water as needed. After 14 days, leaves (fully expanded primary leaves and first emerging trifolate leaf), cotyledons, hypocotyls and roots were separated and protein samples were extracted from each organ. All tissues (dark- and light-grown plants) were harvested at 09:00 h, 2 h into the light cycle. Tissue samples (200 mg) were ground in 400 μl of buffer containing (40 mM Tris–HCl, pH 7.2, 1 mM EDTA, 5% (v/v) glycerol and 0.01 mg ml^{-1} bromophenol blue). Triton X-100 was added to each sample to a final concentration of 0.05% (v/v), to disrupt organelles. To test the effects of PVP-40 and/or β-mercaptoethanol (β-ME) on the protein extracts and isoenzyme patterns, they were added to samples at final concentrations of 0.5% (w/v) and/or 5 mM, respectively. Fresh phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM in all extraction buffers. The samples were incubated on ice for 30 min. Debris was removed from the sample by centrifugation at 13000 × *g* for 10 min.

2.2. Enzymatic activity assays

NADH-GDH activity was assayed in 100 mM Tris–HCl, pH 8.0, 0.14 mM NADH and 1 mM $CaCl_2$ with 100 mM NH_4Cl and 10 mM α-ketoglutarate as substrates, and the decrease in absorbance at 340 nm was recorded over 1 min. One unit of GDH activity is defined as the oxidation of 1 μmole of coenzyme (NADH) per min at 30°C.

2.3. Gel electrophoresis, gel staining and immunoblot procedures

GDH isoenzymes in various tissues were resolved by native PAGE (6% polyacrylamide) with an acrylamide:*N*,*N*′-methylene-bis-acrylamide ratio of 125:1, as described by Turano et al. [14], or by native PAGE (4–15% polyacrylamide gradient gels) using a PhastSystem (Pharmacia). Protein bands containing GDH activity were visualized in native polyacrylamide gels by incubation in 100 mM Tris–HCl, pH 9.3, with 50 mM glutamate, 0.5 mM NAD or NADP, 0.25 mM nitroblue tetrazolium, and 0.1 mM phenazine methosulfate for 15–60 min at 37°C [16]. The NAD-GDH stain solution was also used to visualize enzyme activity on nitrocellulose (see Section 2.4). Identical gels were incubated in the GDH stain solution minus glutamate as controls.

Immunoblot analysis was conducted as described by Turano et al. [17]. Proteins were separated by native PAGE as described above, SDS-PAGE in 7.5% polyacrylamide gels as described by Laemmli [18], or by SDS-PAGE in a homogeneous (7.5%) polyacrylamide gel using the PhastSystem (Pharmacia). Rabbit serum raised against grape leaf NAD(H)-GDH was kindly provided by Loulakakis and Roubelakis-Angelakis [4]. Antibodies specific to GDH were purified by immunoaffinity to *Arabidopsis* GDH. Protein samples containing *Arabidopsis* GDH were separated by SDS-PAGE, blotted on to nitrocellulse and incubated with rabbit serum raised against grape leaf NAD(H)-GDH as described by Turano et al. [11]. GDH-specific antibodies were eluted from strips of nitrocellulose by incubation with 0.1 M glycine, pH 2.6, containing 0.15 M NaCl at 22°C for 15 min. The nitrocellulose was removed and the solution was neutralized with 1 M Tris–HCl, pH 8.0. Sodium azide was added to a final concentration of 0.01% (w/v) prior to storage at −70°C.

Due to low activity associated with the NAD(P)-dependent deamination and NADPH-dependent amination reactions and the high background associated with the deamination assays conducted on crude protein extracts [14], only the NADH-dependent reaction was used to determine the amount of activity loaded onto gels. Since we were interested in studying the GDH isoenzyme system and the specific activity in some samples was very low, we chose not to use the amount of protein added per gel as a standard. An equal amount of NADH-GDH activity (0.250 units) was added per lane for native gels in the Mini-Protean II system (Bio-Rad), but less activity (approximately 0.010 units) was added to the PhastStystem gels.

2.4. Tissue print analysis

Tissue print analyses were conducted on 5-day-old dark-grown seedlings to determine tissue-specific accumulation. Nitrocellulose membranes (Schleicher & Schuell) were preincubated in mitochondria lysis buffer containing 10 mM Tris–HCl, pH 7.5, 1 mM EDTA and 0.1% (v/v) Tween 20 [14] for 5 min at room temperature and then air dried. Longitudinal- and cross-sections of hypocotyls and roots were prepared on nitrocellulose as described by Varner [19]. Tissue prints were stained for NAD-GDH activity for 1 h at 37°C in the enzyme activity stain solution described above. Identical tissue prints were incubated in the GDH stain solution minus glutamate as controls. After 1 h, the stain solution was diluted 1:1 with water, and the tissue prints were incubated at 4°C for 48 h, except for negative controls which were stained for 7 days. Nitrocellulose membranes were air-dried and observed using a Wild Zoom Stereomicroscope with a 35-mm camera attached. Tissue prints were photographed using Kodak T Max black and white film.

3. Results

3.1. Characterization of GDH isoenzymes in soybean seedlings germinated in the light or dark

Protein extracts from leaves, cotyledons, hypocotyls and roots from 14-day-old soybeans that were germinated in the light and from cotyledons, hypocotyls and roots of 5-day-old soybeans that were germinated in the dark were separated by native PAGE and visualized with NAD-GDH or NADP-GDH activity stains or by immunodetection (Fig. 1). Four isoenzymes were visualized in gels stained specifically for NAD-GDH activity (Fig. 1A). Identical gels stained under identical conditions except minus glutamate showed no visable staining in the regions of these four bands (data not shown), suggesting all the staining was due to glutamate-dependent activity. An apparently novel isoenzyme, designated GDH0 based on its anodal migration and following the nomenclature of McKenzie and Lees [15], was identified in the leaves of 14-day-old seedlings. GDH1 was identified in the cotyledons of seedlings germinated in the light or dark. GDH2 was observed in the cotyledons of seedlings germinated in the light or dark and in the leaves of 14-day-old seedlings. GDH3, the slowest migrating isoenzyme, was abundant in hypocotyls and roots of seedlings germinated in the light or dark. Very little or no GDH3 was observed in leaves of 14-day-old seedlings. Identical native gels were stained for NADP-GDH activity to determine the pyridine dinucleotide specificity of each isoenzyme (Fig. 1B). GDH0 was visible in leaves of 14-day-old seedlings. GDH1 was identified in the cotyledons of seedlings germinated in the light and dark. Based on the intensities of the stains, GDH0 and GDH1 exhibited the highest NADP-GDH activity, GDH2 demonstrated an appreciable amount of NADP-GDH activity, and GDH3 had little or no NADP-GDH activity. As described above, identical gels stained under identical conditions except minus glutamate showed no visable staining in the regions of these three (GDH0, GHD1 and GDH2) bands (data not shown), suggesting all the staining was due to glutamate-dependent activtiy. Immunoblot analysis was utilized to determine immunological relationship among the soybean GDH isoenzymes and to grape NAD(H)-GDH (Fig. 1C). The most intense cross-reaction with anti-NAD(H)-GDH serum was apparent in samples containing GDH3—i.e. roots or hypocotyls of plants germinated in the light or the dark. GDH2 cross-reacted with the antiserum but with less affinity than GDH3, based on the lower intensity of stain. GDH0 and GDH1 did not cross-react with the antiserum to grape NAD(H)-GDH. Immunoblot analyses of SDS-PAGE (Fig. 1D) revealed that a single polypeptide, at approximately 42 kD, cross-reacted with the rabbit serum raised against grape leaf NAD(H)-GDH in soybean tissues that contained either GDH2 or GDH3. As in the immunoblots of native PAGE, the most intense cross-reaction with anti-NAD(H)-GDH serum was apparent in samples containing GDH3 and less intense samples containing GDH2. In summary, the results from the gel stains and immunblot analyses demonstrate there are at least four distinct NAD(P)-GDH isoenzymes in soybean.

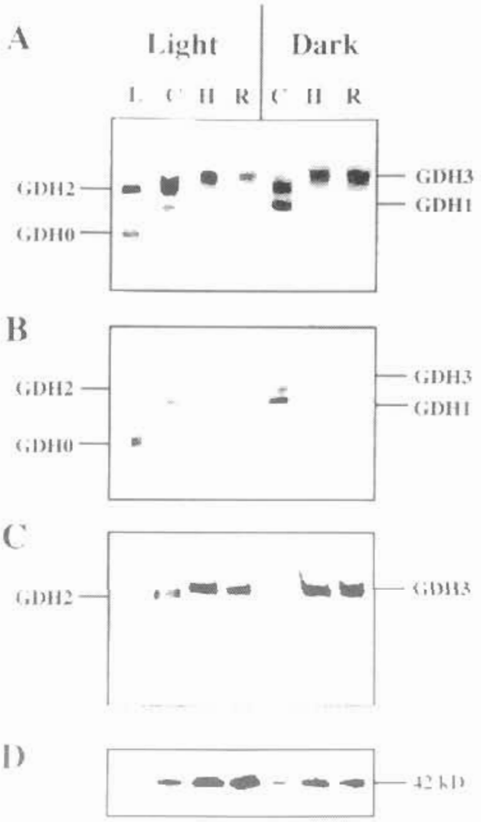

Fig. 1. Characterization of GDH isoenzymes from light- and dark-grown soybean seedlings. Protein extracts from leaves, cotyledons, hypocotyls and roots of 14-day-old soybeans grown in the light or cotyledons, hypocotyls and roots from 5-day-old soybeans germinated in the dark were separated by native PAGE and stained for NAD-GDH (A) or NADP-GDH (B) activity, or immunologically detected (C) with rabbit serum raised against grape leaf NAD(H)-GDH [4]. The four GDH isoenzymes are indicated. Enzyme preparations were analyzed by SDS–PAGE (7.5% polyacrylamide) and immunodetection (D). The 42-kDa polypeptide(s) are indicated.

3.2. Tissue print analysis

Tissue print analysis was used to determine tissue specific activity of GDH in various regions of the axis during germination in the dark. To verify the specificity of the NAD-GDH staining procedure, tissue prints were analyzed with negative controls (NAD-GDH stain minus glutamate) and rabbit serum raised against grape leaf NAD(H)-GDH. Tissue prints for the same four longitudinal- or cross-sections along the axis are presented for the negative controls (Fig. 2) and antiserum to grape NAD(H)-GDH (Fig. 3). The same four sections are presented for the NAD-GDH stain with additional cross-sections for a more detailed analysis (Fig. 4). There were no regions of non-specific NAD-dependent staining in the longitudinal-section of the hypocotyl hook (Fig. 2A), cross-section of the hypocotyl, in the region below the hypocotyl hook and above the root hair zone (Fig. 2C) and cross-sections through the root, in the region of the functional root hairs (Fig. 2D). However, there was a faint reaction in the cross-section of the hypocotyl, in the region of the hook (Fig. 2B). In all cases, there was a physical impression of the print which

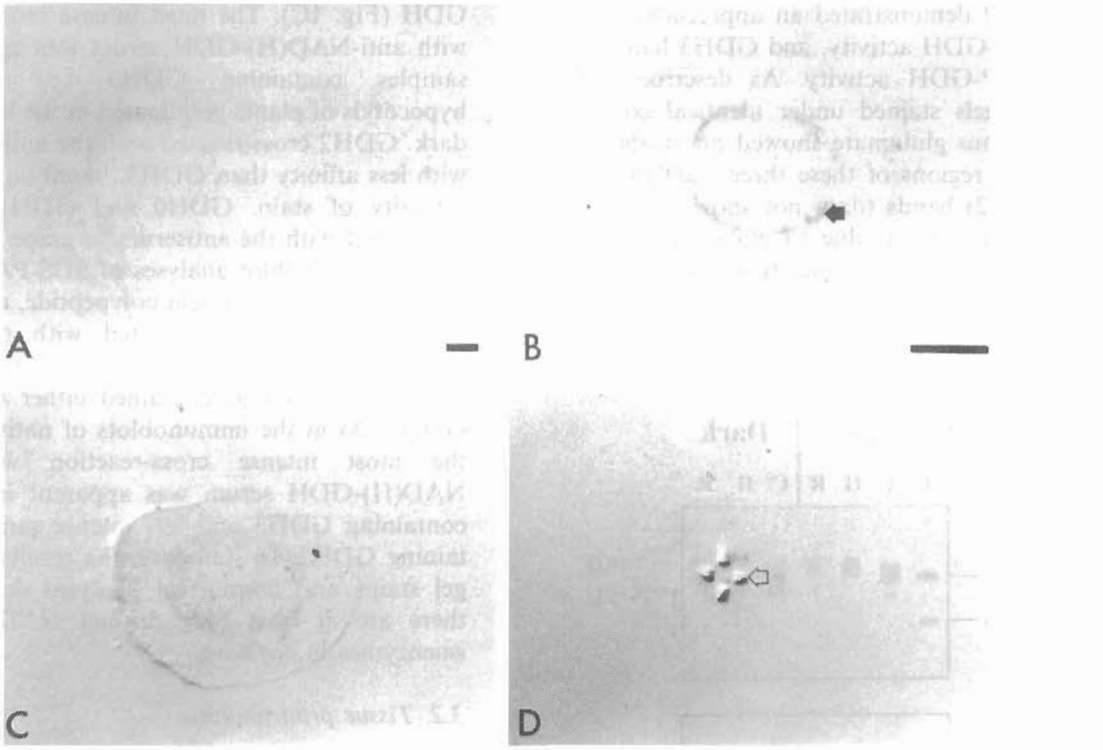

Fig. 2. Tissue print analysis of hypocotyls and roots from soybeans germinated in the dark. Longitudinal section of the hypocotyl (A) and cross-sections of hypocotyl (B,C) and root (D) were blotted to nitrocellulose and stained with NAD-GDH stain minus Glu (see Section 2). The phloem (arrow) or xylem (open arrow) are indicated. The bar equals 0.5 mm. The magnification in panels B–D is the same.

demonstrated that enough pressure was applied to ensure transfer of the proteins and in many cases the impressions provide morphological reference points—i.e. in the root (Fig. 2D) the xylem is clearly visible as four distinct triangular strands as described by Weaver [20].

Antiserum against grape NAD(H)-GDH was used to localize GDH in the axis. The antiserum cross-reacted with proteins throughout the hypocotyl hook (Fig. 3A) with an intense reaction in the vascular tissue and the epidermal layer. The cross-reactivity was visible as a darkly stained area in the phloem and in the epidermal layer of cross-sections in the region of the hypocotyl hook (Fig. 3B). In addition there was a significant amount of staining throughout the cortex. In cross-sections of the hypocotyl in the region below the hook and above the root hair zone, there was strong cross-reaction around the vascular cambium and the epidermal regions with moderate staining throughout the cortex (Fig. 3C). In the region of the functional root hairs (Fig. 3D), there was staining in the epidermal layer and faint stain in the vascular cylinder with some staining in the phloem and no staining in the xylem.

Tissue print analysis was used to determine tissue specific activity of NAD-GDH in various regions of the axis during germination of soybeans (Fig. 4). In a longitudinal-section of the hypocotyl hook (Fig. 4A) there were two prominent regions of NAD-GDH activity, the epidermis and the vascular bundle. Throughout the region of hypocotyl hook, the epidermis stained for NAD-GDH activity (Fig. 4A–D). Specific staining for NAD-GDH activity in the vascular tissue of the hypocotyl was observed by a series of cross-sections (Fig. 4B–D). Throughout the region of the hypocotyl hook, the phloem stained

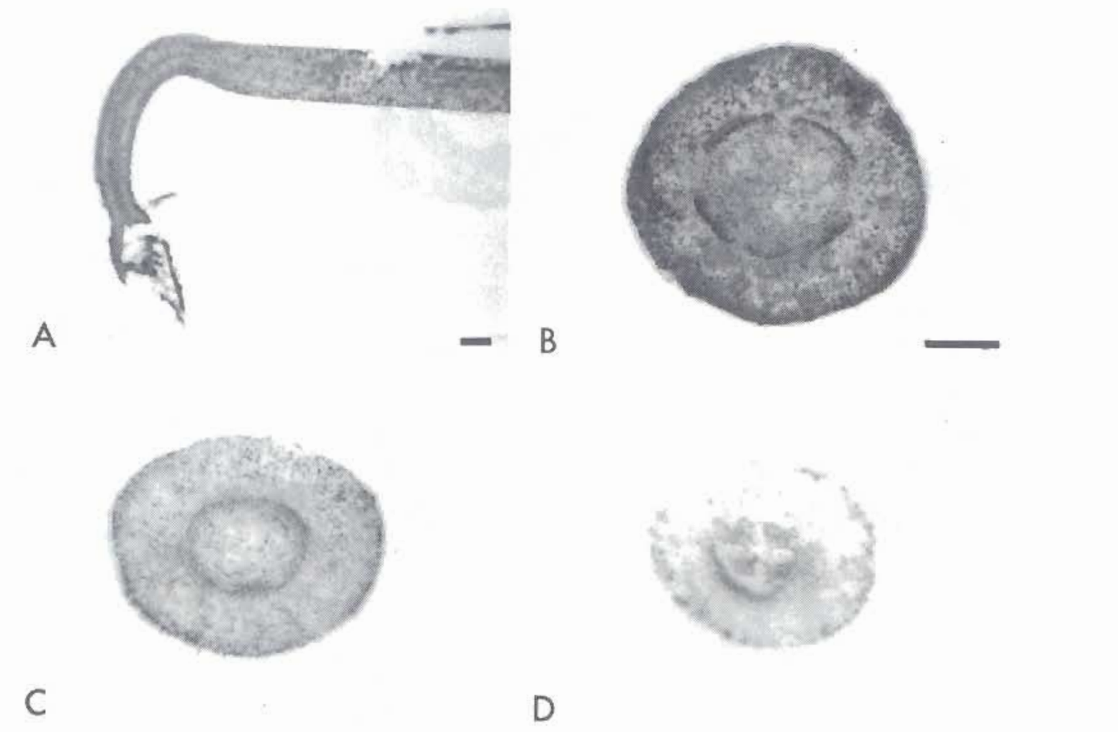

Fig. 3. Immunological localization of NAD(H)-GDH using tissue prints of hypocotyls and roots from soybeans germinated in the dark. Tissue sections were blotted to nitrocellulose and treated with rabbit serum raised against grape leaf NAD(H)-GDH [4]. Longitudinal and cross-sections of the hypocotyl and root are the same as Fig. 2. The bar equials 0.5 mm. The magnification in panels B–D is the same

for NAD-GDH activity. The phloem appeared as eight intensely stained strands that are visible as two hemispheres, in the region of the hook proximal to the cotyledons (Fig. 4B). As the distance from the cotyledons increased, the phloem was visable as four (Fig. 4C) and six (Fig. 4D) intensely stained strands in the form of a circle. In a cross-section of the hypocotyl, in the region below the hook and above the root hairs, the region similar to Fig. 2C and Fig. 3C, there was intense NAD-GDH staining around the vascular cambium and the epidermal regions with moderate staining throughout the cortex (Fig. 4E). In the region of the hypocotyl, proximal to the root, there was intense staining throughout the cortex and the inner region of the vascular cylinder, however the vascular tissue, phloem and xylem, were devoid of NAD-GDH activity (Fig. 4F). In cross-sections throughout the root (Fig. 4G,H), NAD-GDH activity was localized to the epidermal layer. Throughout the root, all other tissue types, including the xylem, phloem, endodermis and cortex, were void of NAD-GDH activity. In the region of the root above the functional root hairs, a physical impression of the xylem was clearly visible as four distinct lines (Fig. 4G). Likewise, a physical impression of the xylem was clearly visible as four distinct triangular strands (Fig. 4H) in the region of the functional root hairs. In a cross-section of an emerging lateral root (Fig. 4I), NAD-GDH activity was evident in the growing root tip and in the epidermis.

4. Discussion

Four GDH isoenzymes were identified in protein extracts from different soybean organs. In

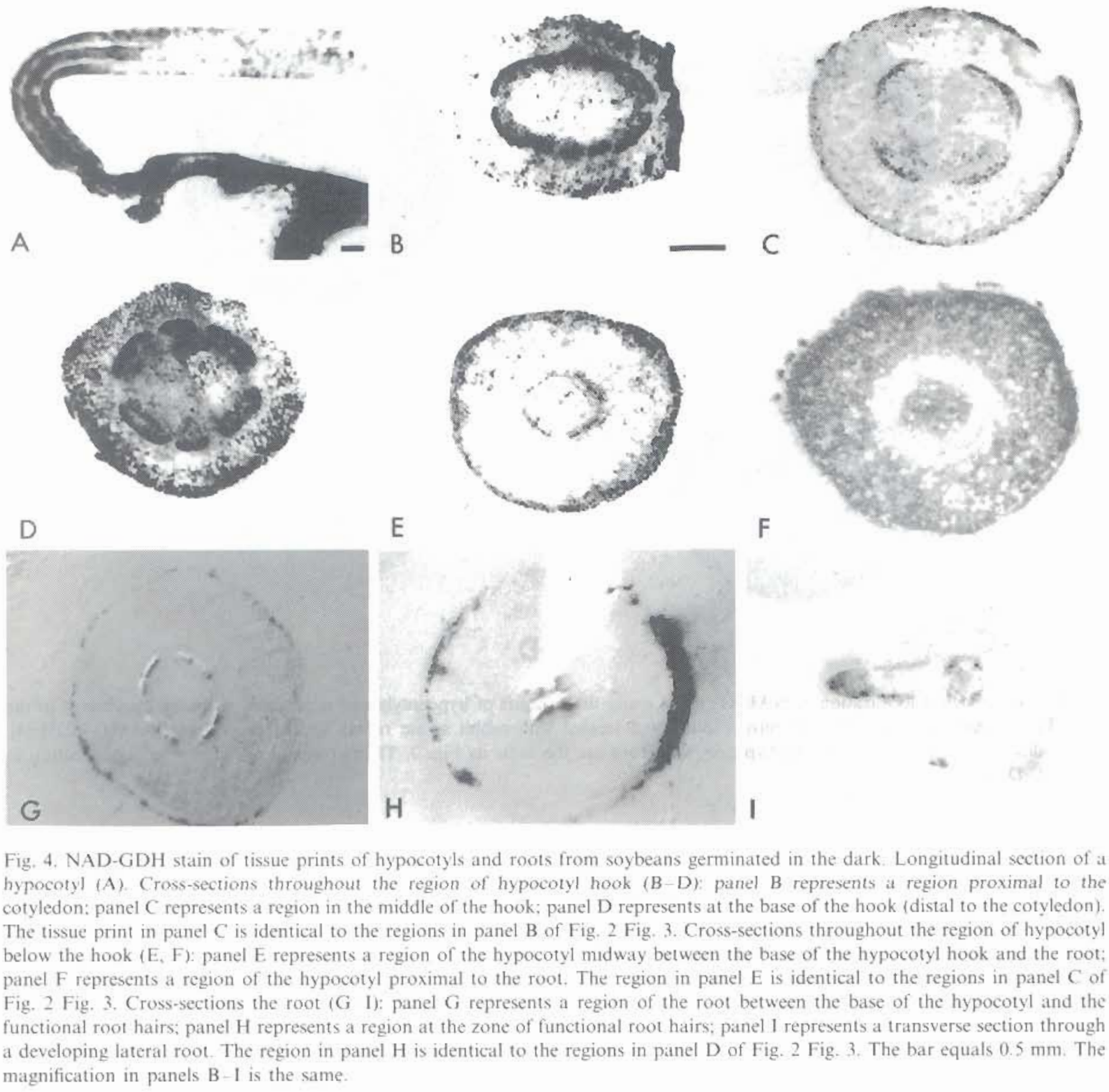

Fig. 4. NAD-GDH stain of tissue prints of hypocotyls and roots from soybeans germinated in the dark. Longitudinal section of a hypocotyl (A). Cross-sections throughout the region of hypocotyl hook (B–D): panel B represents a region proximal to the cotyledon; panel C represents a region in the middle of the hook; panel D represents at the base of the hook (distal to the cotyledon). The tissue print in panel C is identical to the regions in panel B of Fig. 2 Fig. 3. Cross-sections throughout the region of hypocotyl below the hook (E, F): panel E represents a region of the hypocotyl midway between the base of the hypocotyl hook and the root; panel F represents a region of the hypocotyl proximal to the root. The region in panel E is identical to the regions in panel C of Fig. 2 Fig. 3. Cross-sections the root (G–I): panel G represents a region of the root between the base of the hypocotyl and the functional root hairs; panel H represents a region at the zone of functional root hairs; panel I represents a transverse section through a developing lateral root. The region in panel H is identical to the regions in panel D of Fig. 2 Fig. 3. The bar equals 0.5 mm. The magnification in panels B–I is the same.

previous studies, three of the isoenzymes were observed in native gels stained specifically for NAD-GDH [15,14]. In this study, an additional GDH isoenzyme was identified. The isoenzyme migrated faster than any of the previously reported soybean NAD-GDH isoenzymes. Based on its anodal migration and following the nomenclature system of McKenzie and Lees [15], the isoenzyme was designated GDH0. GDH0 utilized both NAD and NADP. However, based on the different staining intensities, it appeared to have greater activity with NADP than NAD. However this would have to be examined in the future with specific kinetic analyses. GDH0 was previously undetected for one of the following reasons: (1) the tissues that were used in earlier studies [13–

15] did not have detectable amounts of GDH0, (2) the gel system used by McKenzie and Lees [15] may not have resolved GDH0, and/or (3) the tissue samples from earlier studies were treated in a manner such that GDH0 became inactive. We have only detected GDH0 in immature primary leaves (this report) and in mature soybean seeds but not in nodules, roots, stems, petioles, fully expanded leaves, flowers, pods, or developing seeds (data not shown); therefore, the first explanation may provide a plausible reason of why GDH0 was previously undetected. GDH1 from cotyledons utilized both NAD or NADP. Based on the intensity of the staining, it appeared that GDH1 may prefer NADP over NAD; these findings are consistent with the findings of McKenzie and Lees [15]. GDH2 appeared to prefer NAD over NADP. McKenzie et al. [13] suggested that GDH2 showed some NADP activity. GDH3 appeared to be NAD specific; these findings are consistent with our earlier findings [14] and those of McKenzie et al. [13] which demonstrated that GDH3 preferentially utilized NAD(H) over NADP(H).

Other than the appearance of GDH0 in leaves of plants maintained in the light, the most prominent difference in isoenzyme patterns was observed in the cotyledons of light versus dark treated plants. In the cotyledons of light versus dark treated plants, the intensity of both the NAD-GDH and NADP-GDH staining for GDH2 appeared unchanged. However, both the NAD-GDH and NADP-GDH staining for GDH1 was less intense in the cotyledons of plants maintained in the light versus those maintained in the dark.

Results from immunoblot analysis suggest that GDH2 and GDH3 are immunologically related, since the two isoforms cross-reacted with the rabbit serum raised against grape leaf NAD(H)-GDH. GDH0 and GDH1 did not cross-react with the rabbit serum raised against grape leaf NAD(H)-GDH, suggesting these isoenzymes are immunologically distinct from GDH2 and GDH3. The cross-reactivity of the NAD(H)-GDH antiserum was correlated with the cofactor activity of the isoenzymes: i.e. GDH2 and GDH3 stained more intensely with NAD than NADP and cross-reacted with the antiserum, while GDH0 and GDH1, which stained more intensely with NADP than NAD, did not cross-react with the antiserum. These findings are consistent with our findings in *Arabidopsis* where NAD(H)-GDH isoenzymes are immunologically and genetically distinct from the NADP(H)-GDH isoenzyme(s) (F.J. Turano, unpublished results). Furthermore, based on the intensity of the stain, the antiserum to grape NAD(H)-GDH had a higher affinity for GDH3 than for GDH2, suggesting GDH3 has higher similarity to the grape isoenzyme than GDH2.

The distinctive electrophoretic mobilities and the different immunoaffinity with antiserum to grape NAD(H)-GDH of the soybean GDH isoenzymes could be explained by differential post-translational modification of the isoenzymes or by the presence of distinct gene(s) encoding for each isoenzyme. To date there are no data to suggest that plant GDH isoenzymes are post-translationally modified, but there are conclusive data to show that some plant GDH isoenzymes are encoded by distinct genes [11]. Based on our present knowledge of GDH isoenzymes systems in plants, it appears that there may be several GDH genes in soybean; however, since the soybean GDH isoenzyme system does appear to be different (see below) than those of other plants studied to date, post-translational modification of the isoenzymes can not be ruled out at this time.

In this study, four NAD(H)-GDH isoenzymes, either GDH0, GDH1, GDH2 or GDH3, were identified in different soybean tissues. The soybean NAD-GDH isoenzymes do not resolve into seven distinct bands as in *Arabidopsis* [8], avocado [12], grape [3], pea [21] and maize [22]. The inability to resolve the NAD-GDH isoenzymes into seven distinct bands by native PAGE could be due to one of several factors: (1) the use of an inappropriate gel concentration, (2) degradation of the proteins by proteases, and/or (3) altered integrity of the proteins due to impurities in the extracts. Numerous attempts to improve the resolution of the soybean NAD-GDH isoenzymes by altering the native and isoelectric focusing PAGE systems were made but with limited success (for details see [14]). Precautions were taken throughout this, and earlier [14], investigation(s) to protect proteins from protease activity by adding fresh PMSF to the extraction buffer and maintaining the samples on ice. To maintain the integrity of the isoenzymes, PVP and/or β-ME were added to the protein extracts. The migration of the isoenzymes were not altered nor was the resolution of the enzymes improved with the addition of either PVP and/or β-ME. However, the addition of β-ME may preserve NADP(H)-GDH activity (data not shown). Based on the results form this and earlier studies [13–15], it appears that the soybean GDH isoenzyme system is different than the other GDH isoenzyme systems mentioned above.

Immunoblot analyses of SDS-PAGE revealed that a single polypeptide, at approximately 42 kDa, cross-reacted with the rabbit serum raised against grape leaf NAD(H)-GDH in soybean tissues that contained either GDH2 or GDH3. The estimated relative molecular mass of GDH3 in a native PAGE is approximately 280 kDa [14]. GDH2 migrates slightly faster in a native gel than GDH3 and is therefore slightly less than 280 kDa. Based on these observations, GDH2 and GDH3 appear to be hexamers composed of single peptides with very similar or identical molecular size. These results suggest that the architecture of soybean NAD(H)-GDH isoenzymes may be different than those of grape [5], avocado [12], and Arabidopsis [11]. In those plants, NAD(H)-GDH isoenzymes are composed of subunits of different molecular size between approximately 42 and 43 kDa that can be resolved by SDS-PAGE. The two subunits combine in different ratios to form seven enzymatically active hexameric complexes. The absence of subunits with different molecular weights in soybean may provide an explanation of why soybean NAD(H)-GDH isoenzymes do not resolve into seven distinct bands in native polyacrylamide gels like other plant NAD(H)-GDH isoenzymes. However, it is possible that other NAD(H)-GDH subunits, which can not be readily detected with the antiserum to grape NAD(H)-GDH due to their low abundance in soybean and/or low affinity with the antiserum, exist in soybean.

Since GDH3 was detected in the roots and hypocotyls in native gels stained for GDH activity and by Western blot analysis to the exclusion of GDH0, GDH1, and GDH2, tissue prints in conjunction with NAD-GDH activity staining procedures and immunological techniques were used to demonstrate tissue-specific activity of GDH3 throughout the axis (root and hypocotyl). Other dark- or light-grown samples were not amenable to tissue print analyses due to the presence of multiple isoenzymes (i.e. cotyledons), physical constrains associated with actual process of tissue printing (i.e. leaves) and/or the specific activity was too low to detect activity or GDH peptide by immunodetection in the tissues (i.e. hypocotyls of light-grown plants). Pretreatment of the nitrocellulose membrane with detergent (see Section 2) was absolutely necessary to obtain maximal NAD-GDH staining. Since GDH3 was localized in the mitochondria [14], it is plausible that the detergent disrupted the mitochondrial membrane, liberated the enzyme, and enabled the protein to bind to the nitrocellulose membrane. Tissue print analysis was useful to determine the tissue-specific activity of GDH3 throughout the axis, but the technique could not be used to specifically identify GDH0, GDH1, or GDH2 in the other organs. Presently, the absence of GDH0, GDH1, or GDH2 in hypocotyl and root can not be entirely disregarded until a more sensitive detection system—i.e. immunodetection with monoclonal antibodies specific to each of the GDH isoenzymes—is developed. Regardless of the limitations on the technique, tissue print analysis proved to be an effective method to demonstrate tissue-specific NAD(H)-GDH activity, most likely GDH3, in the axis. The validity of the techniques was verified in several ways. First, tissue prints incubated in NAD-GDH stain minus glutamate (i.e. negative controls), up to 7 days, had little or no background dehydrogenase activity (Fig. 2). Second, the activity of selected dehydrogenases, homoserine dehydrogenase and glucose-6-phosphate dehydrogenase, alcohol dehydrogenase and malate dehydrogenase (data not shown) were distinct from that of NAD-GDH (Figs. 3 and 4). Third, immunological detection of GDH on the tissue prints (Fig. 3) was consistent with the

NAD-GDH staining (Fig. 4). Fourth, GDH activity that was observed on the prints appeared genuine and was not a result of uneven transfer of proteins to the nitrocellulose due to variations of contact between the tissue and the membrane. This was best exemplified on prints where physical imprints of anatomical or morphological features were visible on the nitrocellulose membrane but were devoid of NAD-GDH activity (Fig. 4G, H). An equally strong example of the specificity of the NAD-GDH staining was exhibited on the same prints where areas devoid of any physical impression contained intense NAD-GDH activity.

Immunaffinity purified antibodies to grape NAD(H)-GDH were used in the tissue print analysis to avoid problems associated with not specific binding. When these antibodies were used for immunoblot analysis, they cross-reacted with soybean GDH isoenzymes with little or no background. However, background on the tissue prints visualized by immunodetection is slightly higher than that visualized by GDH staining, but the GDH activity and immunolocalization do correlate very well. The slight variation in the results may reflect differences in the sensitivities between the two techniques—i.e. immunodectetion being more sensitive, and/or differences between the detection of active (both immunodetection and activity stain) versus inactive (only immunodetection) GDH isoenzyme(s) on the prints. Experiments with preimmune serum from rabbits exhibited no cross-reaction (data not shown).

The most striking observation in the tissue print analyses were changes in NAD-GDH activity throughout the vascular tissue, specifically the phloem. NAD-GDH activity was clearly evident in phloem in the region of the hypocotyl hook (Fig. 4A–D) and the mid-region of the hypocotyl (Fig. 4E), whereas there was very little or no GDH activity in the phloem throughout the lower hypocotyl region (Fig. 4F) and the root (Fig. 4G,H). These results suggest that GDH3 may play a specific role in the transport of nutrients (i.e. carbon and nitrogen reserves) from the cotyledons in developing soybean seedlings. In *Arabidopsis*, NAD(H)-GDH activity [11] and gene expression [10,11] have been shown to increase when either carbon is limiting or nitrogen is in excess. Since both carbon and nitrogen reserves are transported from the cotyledons during germination, it is not possible to state which compounds, if any, affect NAD(H)-GDH activity in soybean seedlings. If either of the compounds are involved NAD(H)-GDH activation in soybean, then the data suggests that their influence decreases as the distance from the cotyledons increases. Alternatively, NAD(H)-GDH activation or expression may be regulated in a spatial or organ-specific manner throughout the phloem. Tissue print analysis also suggests that GDH3 may play a specific role in carbon and/or nitrogen transport and/or absorption in the epidermis of roots.

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References

[1] G.R. Stewart, A.F. Mann, P.A. Fentem, Enzymes of glutamate formation: glutamate dehydrogenase, glutamine synthetase and glutamate synthase, in: B.J. Miflin (Ed.), The Biochemistry of Plants, vol 5, Academic Press, New York, 1980, pp. 271–327.

[2] H.S. Srivastava, R.P. Singh, The role and regulation of L-glutamate dehydrogenase activity in higher plants, Phytochemistry 26 (1987) 597–610.

[3] K.A. Loulakakis, K.A. Roubelakis-Angelakis, Intracellular localization and properties of NAD(H)-glutamate dehydrogenase from *Vitis vinifera* L.: purification and characterization of the major leaf isoenzyme, J. Exp. Bot. 41 (1990) 1223–1230.

[4] K.A. Loulakakis, K.A. Roubelakis-Angelakis, Immunocharacterization of NADH-glutamate dehydrogenase from *Vitis vinifera* L., Plant Physiol. 94 (1990) 109–113.

[5] K.A. Loulakakis, K.A. Roubelakis-Angelakis, Plant NAD(H)-glutamate dehydrogenase consists of two subunit polypeptides and their participation in the seven isoenzymes occurs in an ordered ratio, Plant Physiol. 97 (1991) 104–111.

[6] K.A. Loulakakis, K.A. Roubelakis-Angelakis, The seven NAD(H)-glutamate dehydrogenase isoenzymes exhibit similar anabolic and catabolic activities, Physiol. Plant. 96 (1996) 29–35.

[7] K.M. Syntichaki, K.A. Loulakakis, K.A. Roubelakis-Angelakis, The amino-acid sequence similarity of plant glutamate dehydrogenase to the extremophilic archaeal enzyme conforms to its stress-related function, Gene 168 (1996) 87–92.

[8] D. Cammaerts, M. Jacobs, A study of the polymorphism and the genetic control of the glutamate dehydrogenase isoenzymes in *Arabidopsis thaliana*, Plant Sci. Lett. 31 (1983) 65–73.

[9] D. Cammaerts, M. Jacobs, A study of the role of glutamate dehydrogenase in nitrogen metabolism of *Arabidopsis thaliana*, Planta 163 (1985) 517–526.

[10] R. Melo-Oliveira, I.C. Oliveira, G.M. Coruzzi, *Arabidopsis* mutant analysis and regulation define a nonredundant role for glutamate dehydrogenase in nitrogen assimilation, Proc. Natl. Acad. Sci. USA 93 (1996) 4718–4723.

[11] F.J. Turano, S.S. Thakkar, T. Fang, J.M. Weisemann, Characterization and expression of NAD(H)-dependent glutamate dehydrogenase genes in *Arabidopsis thaliana*, Plant Physiol. 113 (1997) 1329–1341.

[12] K.A. Loulakakis, K.A. Roubelakis-Angelakis, K.A. Kanellis, Regulation of glutamate dehydrogenase and glutamine synthetase in avocado fruit during development and ripening, Plant Physiol. 106 (1994) 217–222.

[13] E.A. McKenzie, L. Copeland, E.M. Lees, Glutamate dehydrogenase activity in developing soybean seed: kinetic properties of three forms of the enzyme, Arch. Biochem. Biophys. 212 (1981) 290–297.

[14] F.J. Turano, R. Dashner, A. Upadhyaya, C.R. Caldwell, Purification of mitochondrial glutamate dehydrogenase from dark grown soybean seedlings, Plant Physiol. 112 (1996) 1357–1364.

[15] E.A. McKenzie, E.M. Lees, Glutamate dehydrogenase activity in developing soybean seed: isolation and characterization of three forms of the enzyme, Arch. Biochem. Biophys. 212 (1981) 298–305.

[16] T. Hartmann, M. Nagel, H.J. Ilert, Organ-specific multiple forms of glutamate dehydrogenase in *Medicago sativa*, Planta 111 (1973) 119–128.

[17] F.J. Turano, R.L. Jordan, B.F. Matthews, Immunological characterization of in vitro forms of homoserine dehydrogenase from carrot suspension cultures, Plant Physiol. 92 (1990) 395–400.

[18] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[19] J.E. Varner, Tissue printing demonstration, in: P.D. Reid, R.F. Pont-Lezica, E. Del Campillo, R. Taylor (Eds.), Tissue Printing: Tools for the Study of Anatomy, Histochemistry, and Gene Expression, Academic Press, New York, 1992, pp. 4–9.

[20] H.L. Weaver, Vascularization of the root hypocotyl cotyledon axis of *Glycine max* (L.) Merrill, Photomorphology 10 (1960) 82–86.

[21] H.-W. Scheid, A. Ehmke, T. Hartmann, Plant NAD-glutamate dehydrogenase. Purification, molecular properties and ion activation of the enzymes from *Lemna minoris* and *Pisum sattivum*, Plant J. 7 (1979) 61–75.

[22] H. Sakakibara, K. Fuji, T. Sugiyama, Isolation and characterization of a cDNA that encodes maize glutamate dehydrogenase, Plant Cell Physiol. 36 (1995) 789–797.